EXHIBIT C

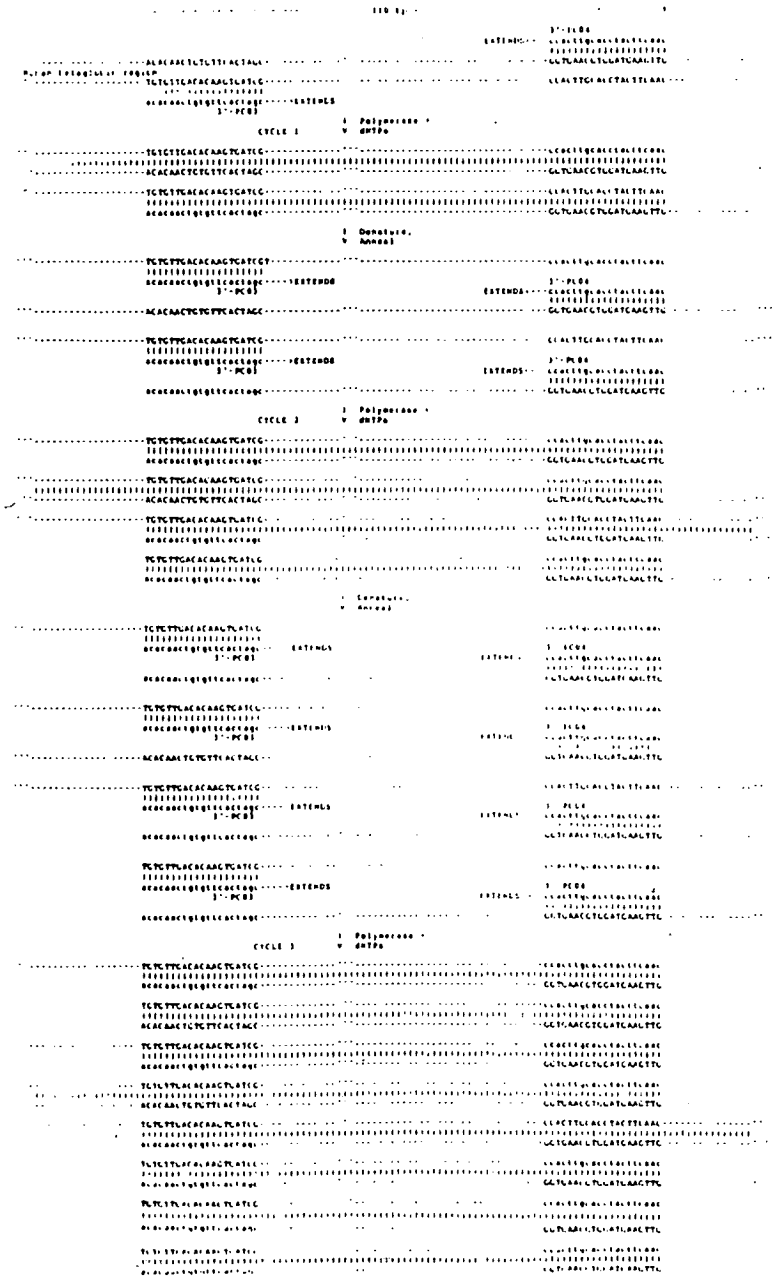
[21] Specific Synthesis of DNA *in Vitro* via a Polymerase-Catalyzed Chain Reaction

By KARY B. MULLIS and FRED A. FALOONA

We have devised a method whereby a nucleic acid sequence can be exponentially amplified *in vitro*. The same method can be used to alter the amplified sequence or to append new sequence information to it. It is necessary that the ends of the sequence be known in sufficient detail that oligonucleotides can be synthesized which will hybridize to them, and that a small amount of the sequence be available to initiate the reaction. It is not necessary that the sequence to be synthesized enzymatically be present initially in a pure form; it can be a minor fraction of a complex mixture, such as a segment of a single-copy gene in whole human DNA. The sequence to be synthesized can be present initially as a discrete molecule or it can be part of a larger molecule. In either case, the product of the reaction will be a discrete dsDNA molecule with termini corresponding to the 5' ends of the oligomers employed.

Synthesis of a 110-bp fragment from a larger molecule via this procedure, which we have termed polymerase chain reaction, is depicted in Fig. 1. A source of DNA including the desired sequence is denatured in the presence of a large molar excess of two oligonucleotides and the four deoxyribonucleoside triphosphates. The oligonucleotides are complementary to different strands of the desired sequence and at relative positions along the sequence such that the DNA polymerase extension product of the one, when denatured, can serve as a template for the other, and vice versa. DNA polymerase is added and a reaction allowed to occur. The reaction products are denatured and the process is repeated until the desired amount of the 110-bp sequence bounded by the two oligonucleotides is obtained.

During the first and each subsequent reaction cycle extension of each oligonucleotide on the original template will produce one new ssDNA molecule of indefinite length. These "long products" will accumulate in a linear fashion, i.e., the amount present after any number of cycles will be linearly proportional to the number of cycles. The long products thus produced will act as templates for one or the other of the oligonucleotides during subsequent cycles and extension of these oligonucleotides by polymerase will produce molecules of a specific length, in this case, 110 bases long. These will also function as templates for one or the other of the oligonucleotides producing more 110-base molecules. Thus a chain reac-



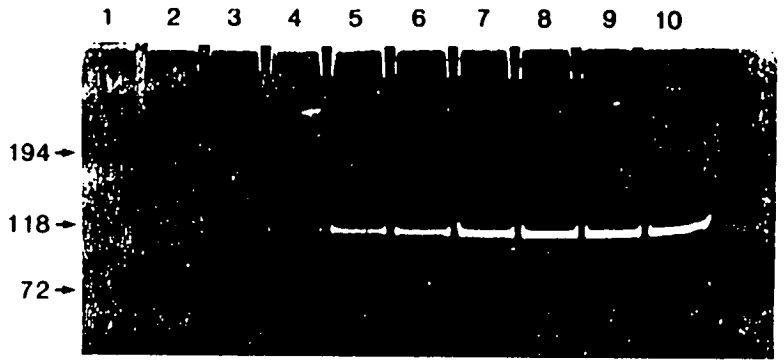
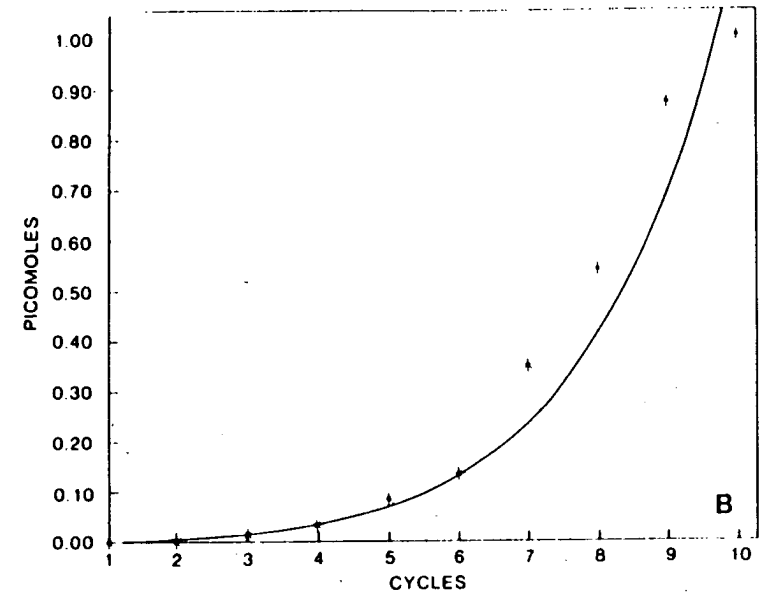


FIG. 2. (A) Reactions were performed as in Method I. DNA target was pBR328::βA, oligonucleotides were PC03 and PC04 at 10 μM, and dNTPs were labeled with α-^{32}P at 500 Ci/mol. After each synthesis cycle 10-μl aliquots were removed and these (lanes 1–10) were analyzed on a 14% polyacrylamide gel in 90 mM Tris–borate and 2.5 mM EDTA at pH 8.3 and 24 V/cm for 2.5 hr. The completed gel was soaked 20 min in the same buffer with the addition of 0.5 μg/ml ethidium bromide, washed with the original buffer, and photographed in UV light using a red filter. The numbers on the left margin indicate the sizes of DNA in base pairs. (B) The 110-bp fragment produced was excised from the gel under UV light and the incorporated ^{32}P counted by Cerenkov radiation. An attempt to fit the data to an equation of the form pmol/10 μl = $0.01[(1 + y)^N - yN - 1]$, where N represents the number of cycles and y the fractional yield per cycle, was optimal with $y = 0.619$. (C) The 8-μl aliquots from the tenth cycle of a reaction similar to the above were subjected to restriction analysis by addition of 1 μl BSA (25 mg/ml) and 1 μl of the appropriate enzyme (undiluted, as supplied by the manufacturer); reacted at 37° for 15 hr; PAGE was performed as above. (1) 1 μg ϕX174/*Hae*III digest, (2) no enzyme, (3) 8 units *Hin*fI, (4) 0.5 units *Mnl*I, (5) 2 units *Msp*I, (6) 3.5 units *Nco*I. The numbers on the left margin indicate the sizes (in base pairs) of DNA.

tion can be sustained which will result in the accumulation of a specific 110-bp dsDNA at an exponential rate relative to the number of cycles.

Figure 2 demonstrates the exponential growth of the 110-bp fragment beginning with 0.1 pmol of a plasmid template. After 10 cycles of polymerase chain reaction, the target sequence was amplified 100 times. The data have been fit to a simple exponential curve (Fig. 2B), which assumes that the fraction of template molecules successfully copied in each cycle remains constant over the 10 cycles. This is probably not true; however, the precision of the available data and our present level of sophistication in fully understanding the several factors involved do not seem to justify a more elaborate mathematical model. This analysis results in a calculated yield per cycle of about 62%. Amplification of this same 110-bp fragment

FIG. 1. The polymerase chain reaction amplification of a 110 bp fragment from the first exon of the human β-globin gene.



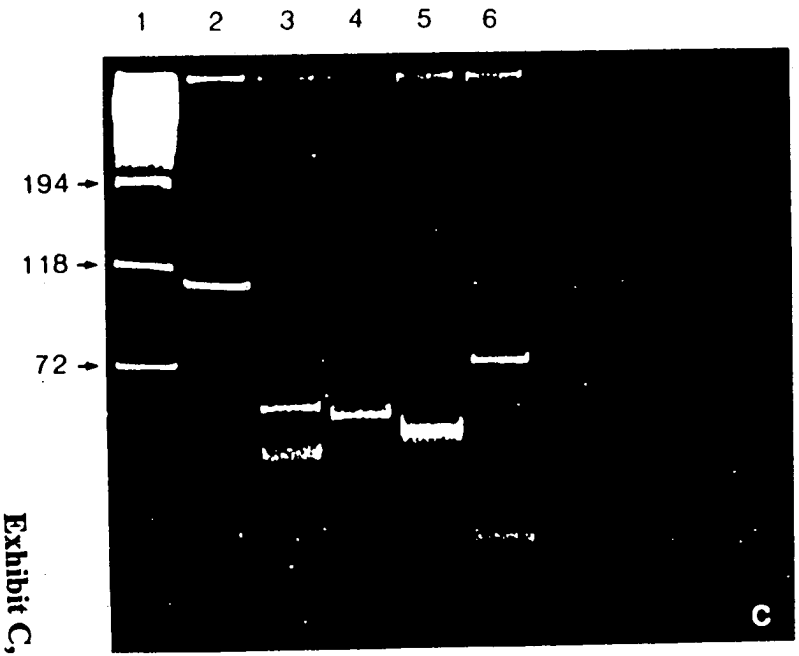


FIG. 2 (*continued*). See legend on p. 337.

starting with 1 μg total human DNA (contains approximately 5×10^{-9} mol of the target sequence from a single-copy gene) produced a 200,000-fold increase of this fragment after 20 cycles. This corresponds to a calculated yield of 85% per cycle.[1] This yield is higher than that in the first example in which the target sequence is present at a higher concentration. It is likely that when the target DNA is present in high concentrations, rehybridization of the amplified fragments occurs more readily than their hybridization to primer molecules.

Materials and Methods

Oligonucleotides were synthesized using an automated DNA synthesis machine (Biosearch, Inc., San Rafael, California) using phosphoramidite chemistry. Synthesis and purification were performed according to the directions provided by the manufacturer.

Oligodeoxyribonucleotides	Designed to produce	From template
EF02 CGCATTAAAGCTTATCGATG	75 bp with EF03	pBR322
EF03 TAGGCGTATCACGAGGCCCT		
EF05 CTTCCCCATCGGTGATGTCG	500 bp with EF03	pBR322
EF05 CCAGCAAGACGTAGCCCAGC	1000 bp with EF03	pBR322
KM29 GGTTGGCCAATCTACTCCCAGG		
KM30 TAACCTTGATACCAACCTGCCC	240 bp with KM29	Globin DNA
KM38 TGGTCTCCTTAAACCTGTCTT	268 bp with KM29	Globin DNA
KM47 AATTAATACGACTCACTATAGGGAGA-TAGGCGTATCACGAGGCCCT	As EF03 plus 26 bp	pBR322
PC03 ACACAACTGTGTTCACTAGC		
PC04 CAACTTCATCCACGTTCACC	110 bp with PC03	Globin DNA
PC05 TTTGCTTCTGACACAACTGTGTTCACTAGC		
PC06 GCCTCACCACCAACTTCATCCACGTTCACC	130 bp with PC05	Globin DNA
PC07 CAGACACCATGGTGCACCTGACTCCTG		
PC08 CCCCACAGGGCAGTAACGGCAGACTTCTCC	58 bp with PC07	Globin DNA

Plasmid pBR328::BA, containing a 1.9-kb insert from the first exon of the human β-globin A allele, and pBR328::βS, representing the β-globin S allele, were kindly provided by R. Saiki.

Restriction enzymes were purchased from New England Biolabs, Beverly, Massachusetts. Klenow fragment of *Escherichia coli* DNA polymerase was purchased from United States Biochemical Corp., Cleveland,

[1] R. Saiki, S. Scharf, F. Faloona, K. Mullis, G. Horn, H. Erlich, and N. Arnheim, *Science* **230**, 1350 (1985).

Ohio, and was the product of a Klenow fragment clone rather than an enzymatic cleavage of DNA polymerase I.

Acrylamide was from Bio-Rad Laboratories, Richmond, California; deoxyribonucleoside triphosphates were from Sigma Chemical Co., St. Louis, Missouri.

NuSieve agarose was purchased from FMC Corporation. Gels were prepared by boiling the appropriate amount of agarose in 90 m*M* Tris–borate at pH 8.3, 2.5 m*M* in EDTA, and containing 0.5 μg/ml ethidium bromide. Poured into horizontal trays, the gels were ~0.5 cm thick, 10 cm long, and were run for 60–90 min at 10 V/cm submerged in the buffer described above. From 4 to 6% NuSieve agarose gels provide separations comparable to 10–15% polyacrylamide; they are considerably easier to cast and load and can be monitored while running with a hand-held UV light. Prior to photography, gels are soaked in water for 20 min to remove unbound ethidium bromide.

The following method is representative of a number of PCR protocols which have been successfully utilized. Specific variations on this procedure are noted in the figure legends and several are summarized below.

Polymerase Chain Reaction: Method I

Dissolve 0.1 pmol pBR322 (1 nM) and 300 pmol each of oligonucleotides FF02 and FF03 (3 μ*M*) (see Diagram 1), and 150 nmol of each deoxynucleoside triphosphate (1.5 m*M*) in 100 μl 30 m*M* Tris–acetate (pH 7.9), 60 m*M* sodium acetate, 10 m*M* dithiothreitol, and 10 m*M* magnesium acetate. The solution is brought to 100° for 1 min, and is cooled to 25° for 30 sec in a waterbath. Add 1.0 μl containing 5 units of Klenow fragment of

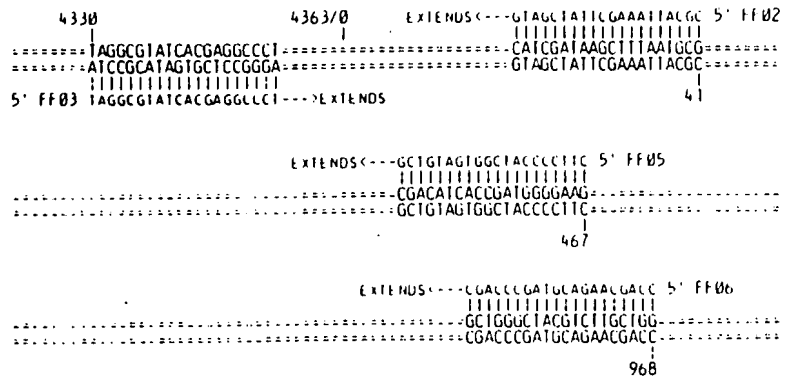


DIAGRAM 1. PCR model systems in pBR322.

E. coli DNA polymerase I and allow the reaction to proceed for 2 min at 25°, after which the cycle of heating, cooling, adding enzyme, and reacting is repeated nine times.

Method I (Summary of Above)

Target DNA: 0.1 pmol
Oligonucleotides: 3 μ*M*, 20-mers
Buffer: 100 μl 30 m*M* Tris–acetate (pH 7.9) 60 m*M* sodium acetate, 10 m*M* Magnesium acetate, and 10 m*M* DTT
dNTPs: 1.5 m*M*
Enzyme: 5 units Klenow fragment
Cycles: Number: 10
Denaturation: 100°, 1 min
Primer hybridization: 25°, 30 sec
Reaction: 25°, 2 min

Method II (Nested Primer Sets)

Target DNA: 10 μg human DNA (0.5×10^{-5} pmol)
Oligonucleotides: 2 μ*M*, outer set: 20-mers; inner set: 27-mer and 30-mer
Buffer: 100 μl 30 m*M* Tris–acetate (pH 7.9), 60 m*M* sodium acetate, 10 m*M* magnesium acetate, and 10 m*M* DTT
dNTPs: 1.0 m*M*
Enzyme: 2 units Klenow fragment
Cycles: Following 20 cycles of amplification with the outer-set primers, a 10-μl aliquot of this reaction was diluted into a further 100-μl reaction mixture containing the inner-set primers and 10 more cycles were performed.
Denaturation: 100°, 1 min
Primer hybridization: 25°, 1 min
Reaction: 25°, 2 min

Method III[1]

Target DNA: 1 μg to 20 ng human DNA (0.5×10^{-6} to 1×10^{-8} pmol)
Oligonucleotides: 1 μ*M*, 20-mers
Buffer: 100 μl 10 m*M* Tris–chloride (pH 7.5), 50 m*M* sodium acetate, and 10 m*M* magnesium chloride
dNTPs: 1.5 m*M*
Enzyme: 1 unit Klenow fragment
Cycles: Number: 20–25
Denaturation: 95°, 5 min, first cycle

95°, 2 min, subsequent cycles
Primer hybridization: 30°, 2 min
Reaction: 30°, 2 min

Method IV[2]

Target DNA: 1 μg human DNA (0.5×10^{-6} pmol)
Oligonucleotides: 1 μ*M*, 20–28-mers
Buffer: 100 μl 30 m*M* Tris–acetate (pH 7.9), 60 m*M* sodium acetate, 10 m*M* Magnesium acetate
dNTPs: 1.5 m*M*
Enzyme: 1 unit Klenow fragment
Cycles: Number: 20
Denaturation: 95°, 2 min
Primer hybridization: 37°, 2 min
Reaction: 37°, 2 min

Method V[2]

As Method IV except
Buffer: 10% DMSO added to Method IV buffer
Cycles: Number: 27

Method VI[3]

Target DNA: 5 ng human DNA containing target + 250 ng human DNA deleted for target, or 1 μg human DNA containing an unknown amount of HTLV-III viral DNA sequence
Oligonucleotides: 1 μ*M*, 15–18-mers
Buffer: 100 μl 10 m*M* Tris–chloride (pH 7.5), 50 m*M* sodium chloride, and 10 m*M* magnesium chloride
dNTPs: 1.5 m*M*
Enzyme: 1 unit Klenow fragment
Cycles: Number: 20–25
Denaturation: 95°, 2 min
Primer hybridization: 25°, 2 min
Reaction: 25°, 2 min

Specificity of the Amplification Reaction

This process has been employed to amplify DNA segments from 24 to 1000 bp in length using template DNA ranging in purity from a highly

[2] S. Scharf, G. Horn, and H. Erlich, submitted for publication.
[3] S. Kwok, D. Mack, K. Mullis, B. Poiesz, G. Ehrlich, D. Blair, A. Friedman-Kien, and J. J. Sninsky, submitted for publication.

purified synthetic single-stranded DNA to a totally unpurified single-copy gene in whole human DNA. Despite the low stringency of the hybridizations the specificity of the overall reaction is intrinsically high, probably due to the requirement that two separate and coordinated priming events occur at each cycle. Beginning with purified plasmid DNA as initial template and pairs of primers intended to produce fragments in the range of 200 bp or less, homogeneous products have usually been observed. Using similar templates, but primers chosen to amplify larger fragments, longer reaction times are required and considerable production of DNA fragments other than that intended is observed (Fig. 3). These by-products are usually smaller than the intended product and can be accounted for by "mispriming" events wherein the 3′ end of one of the primers interacts with a region of partial homology within the sequence of the primary product (see Diagram 2). The probability for synthesis of a by-product representing a subfragment of the primary product is higher than the probability for synthesis of a by-product representing some different sequence in the original reaction for two reasons. First, the concentration of the primary product becomes relatively high during the reaction; and second, any single "mispriming" on a molecule of primary product will result in the production of a new molecule, which like the primary product will contain two primer sites. (A primer "site" in this context would be either a region complementary to one of the primers or a region containing one of the primers, which would in successive cycles produce a sequence complementary to it.) The synthesis of multiple DNA fragments is thus more likely if the intended fragment is large and the final desired concentration of the product is high. The ~225-bp by-product of the amplification of a 500-bp fragment from pBR322 depicted in Fig. 3B can be ac-

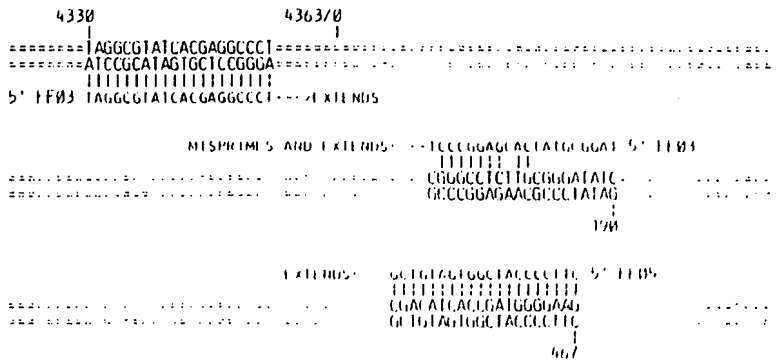


DIAGRAM 2. Probable second priming site on pBR322 for EF03.

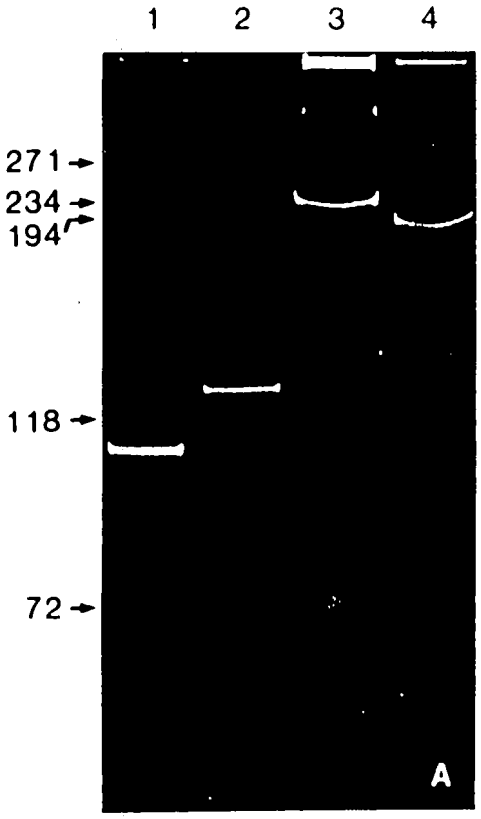


FIG. 3. (A) Reactions were performed as in Method I. DNA target was pBR328::βA, oligonucleotides were (1) PC03 and PC04, (2) PC05 and PC06, (3) KM29 and KM38 (reaction time was 20 min), (4) KM29 and KM30; DNA target was pBR328::βS digested with *Mst*II prior to the reaction. This plasmid is cut several times by *Mst*II but not within the sequence to be amplified by KM29 and KM30. A similar reaction with pBR328::βA which is cut within the target sequence yields no amplified product. The numbers on the left margin indicate the sizes (in base pairs) of DNA. (B) Reactions were performed as in Method I, except reaction times were 20 min per cycle at 37°. Oligonucleotides were FF03 and FF05. Final product was rehybridized for 15 hr at 57°. Electrophoresis was on a 4% NuSieve agarose gel. The numbers on the left margin indicate the sizes (in base pairs) of DNA. (C) (1) Reactions were performed as in Method I. Oligonucleotides were FF02 and FF03. The tenth reaction cycle was terminated by freezing and an 8-μl aliquot was applied to a 4% NuSieve agarose gel visualized with ethidium bromide. (2) Reactions were the same as in (1) except that the oligonucleotides used were FF02 and KM47, which were designed to produce a 101-bp fragment, 26 nucleotides of which are not present in pBR322. The numbers on the left margin indicate the sizes (in base pairs) of DNA. (D) (1) Reactions were performed as in (B). Oligonucleotides were FF03 and FF06. (2) Same as (1) except that KM47 was substituted for FF03. The numbers on the left margin indicate the sizes (in base pairs) of DNA.

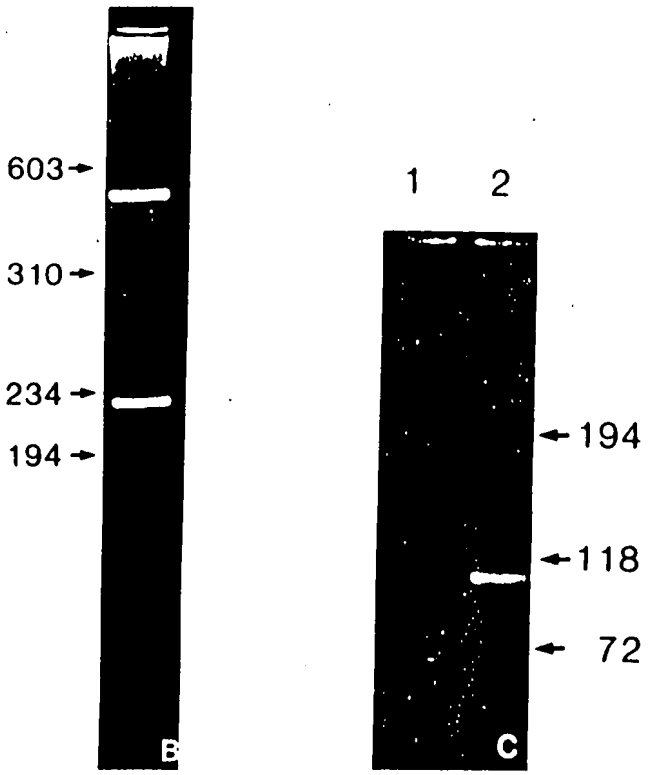


FIG. 3. (*continued*)

counted for by a second priming site for FF03 in which 9 out of 11 of the 3' nucleotides of FF03 find a match within the amplified product.

In Vitro Mutations

"Mispriming" can be usefully employed to make intentional *in vitro* mutations or to add sequence information to one or both ends of a given sequence. A primer which is not a perfect match to the template sequence but which is nonetheless able to hybridize sufficiently to be enzymatically extended will produce a product which contains the sequence of the primer rather than the corresponding sequence of the original template. When this product in a subsequent cycle is template for the second primer the extension product produced will be a perfect match to the first primer

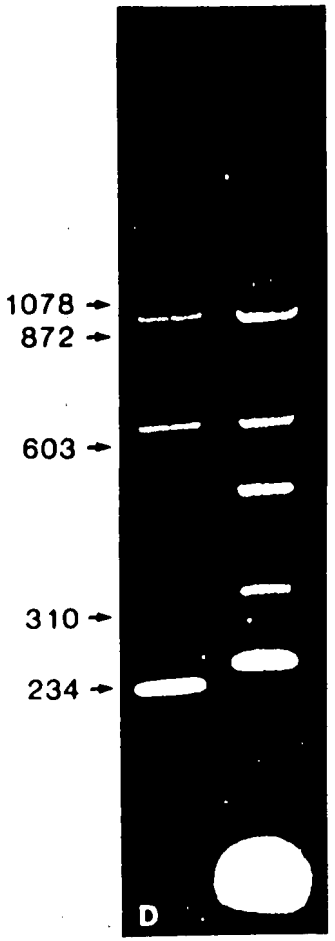


FIG. 3 (*continued*)

and an *in vitro* mutation will have been introduced. In further cycles this mutation will be amplified with an undiminished efficiency since no further mispaired primings are required.

A primer which carries a noncomplementary extension on its 5′ end can be used to insert a new sequence in the product adjacent to the template sequence being copied. In Fig. 3C, lane 2, a 26-bp T7 phage promoter has been appended to a 75-bp sequence from pBR322 by using an oligonucleotide with 20 complementary bases and a 26-base 5′ extension. The procedure required less than 2 hr and produced 2 pmol of the relatively pure 101-bp fragment from 100 fmol of pBR322. Similarly in Fig. 3D, the T7 promoter has been inserted adjacent to a 1000-bp fragment from pBR322.

Scharf *et al.*,[2] in order to facilitate the cloning of human genomic fragments, inserted restriction sites onto the ends of amplified sequences by the use of primers appropriately mismatched on their 5′ ends.

Detection of Minute Quantities of DNA

A microgram of human DNA contains 5×10^{-19} moles of each single-copy sequence. This is ~300,000 molecules. Detection of single-copy sequences in whole human DNA or other similarly complex mixtures of nucleic acids presents a problem which has only been successfully approached using labeled hybridization probes.

Saiki *et al.*,[1] by combining a PCR amplification with a labeled hybridization probe technique, have significantly reduced the time and uncertainty involved in determining the sequence of a single base pair change in the human genome from only a microgram of DNA. They performed a 20-cycle amplification, which required less than 2 hr, and achieved a 200,000-fold increase in the level of a 110-bp sequence in the first exon of the β-globin gene. Once amplified the sequence was relatively simple to analyze.

We attempted to amplify the same 110-bp fragment to a slightly higher level so as to enable visual detection via ethidium bromide staining of a gel. For fragments in this size range, 100 fmol gives rise to a clearly visible band, thus, 0.1 aliquot of a 200,000-fold amplification of 10 μg of human DNA should be sufficient. And so it is; however, control experiments with DNA from a cell line harboring a β-globin deletion indicated that the 110-bp fragment produced was not exclusively representative of the β globin locus. That is, fragments of ~110 bp were being amplified even though no β-globin sequences were present. On the chance that whatever was causing this "background" might not share extensive homology with β-globin in the central 60 nucleotides of this 110-bp region, we attempted to increase the specificity of the process by introducing a second stage of amplification using a second set of primers nested within the first (see Diagram 3). By requiring four separate priming events to take place, we were thus able to amplify approximately 2,000,000-fold and readily detect a β-globin-specific product (Fig. 4).

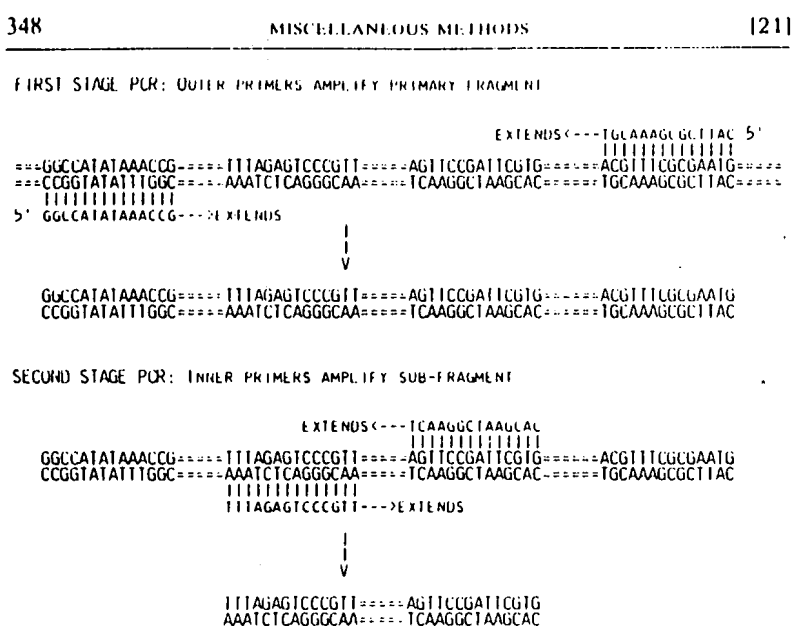


DIAGRAM 3. Nested primer sites, which enable a second layer of specificity. (The sequences here are only examples and have no particular significance.)

The wild-type β-globin allele can be distinguished from the sickle-type allele by the presence of a site for the restriction enzyme *Dde*I. Thus, *Dde*I treatment of the DNA prior to amplification, or of the amplified product subsequent to amplification, will serve to distinguish between these two alleles.

Scharf *et al.*,[3] beginning with 1 μg of human DNA and oligonucleotides 26 and 28 nucleotides in length that were designed to amplify a 240-bp region of the *HLA-DQ-α* gene after 27 cycles of PCR, were able to visualize the predicted fragment via ethidium staining of an agarose gel. In contrast to our results with β-globin, controls with *HLA*-deleted cell lines revealed that this single-stage amplification was specific for the intended target.

Similar amplifications of other human loci have resulted in varying degrees of specificity and efficiency. No simple explanations for this variability, based on, for example, oligomer size, target size, sequence, and temperature, have been forthcoming; however, the number of examples of attempted amplifications of different human sequences is still small.

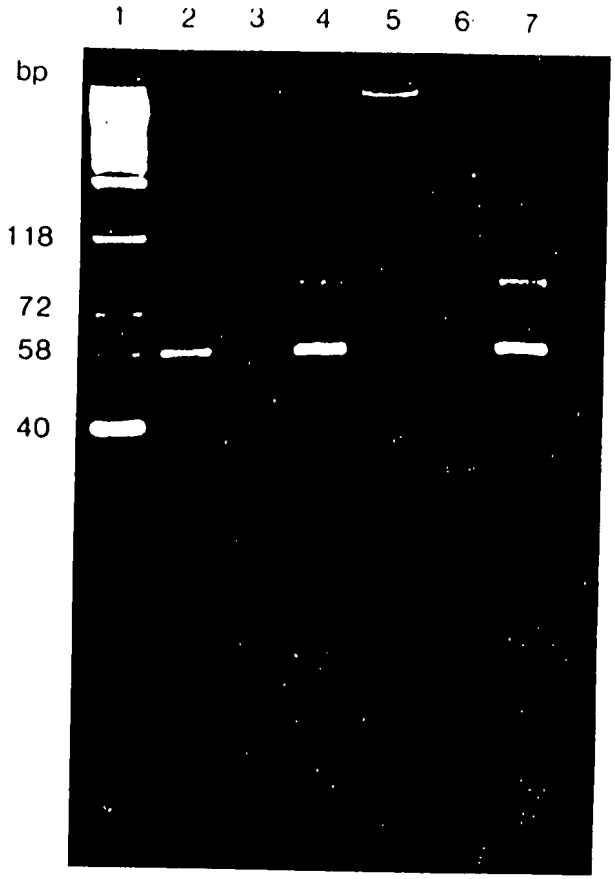


FIG. 4. Reactions were performed as in Method II, and 8-μl aliquots (representing 80 ng of unamplified DNA) were subjected to electrophoresis on a 4% NuSieve agarose gel stained with ethidium bromide. Oligonucleotides were PC03 and PC04, followed by PC07 and PC08 (the nested set). DNA target was as follows: lane (2), human DNA homozygous for the wild-type β-globin allele; lane (3), as in (2) but treated prior to amplification with *Dde*I, which cleaves the intended target and prevents amplification; lane (4), human DNA homozygous for the sickle β-globin allele treated prior to amplification with *Dde*I, which for this allele does not cleave the intended target; lane (5), salmon sperm DNA. Following the final amplification an aliquot of the reaction in (2) was subjected to cleavage with *Dde*I, which should convert the 58-bp wild-type product into 27- and 31-bp fragments [lane (6)]; an aliquot of the reaction in (4) was similarly treated with *Dde*I after amplification [lane (7)]. The 58-bp product from the sickle allele, as expected, contains no *Dde*I site. The numbers on the left margin indicate the sizes (in base pairs) of DNA.

Kwok *et al.*[3] have demonstrated that DNA sequences present at less than one copy per human genome can be successfully amplified and detected. Using an isotopic detection system they were able to identify β-globin sequences in as little as 5 ng of human DNa and have demonstrated sequences of HTLV-III in cell lines derived from patients affected with AIDS.

The polymerase chain reaction has thus found immediate use in developmental DNA diagnostic procedures[1,3] and in molecular cloning from genomic DNA[2]; it should be useful wherever increased amounts and relative purification of a particular nucleic acid sequence would be advantageous, or when alterations or additions to the ends of a sequence are required.

We are exploring the possibility of utilizing a heat-stable DNA polymerase so as to avoid the need for addition of new enzyme after each cycle of thermal denaturation; in addition, it is anticipated that increasing the temperature at which the priming and polymerization reactions take place will have a beneficial effect on the specificity of the amplification.

Acknowledgments

We wish to acknowledge the interest and support of Thomas White, and we would like to thank Corey Levenson, Lauri Goda, and Dragan Spasic for preparation of oligonucleotides; Randy Saiki, Stephen Scharf, Glenn Horn, Henry Erlich, Norman Arnheim, and Ed Sheldon for useful discussions regarding the amplification of human sequences; and Denise Ramirez for assistance with the manuscript.

[22] Visual Assay for Chromosome Ploidy

By DOUGLAS KOSHLAND and PHILIP HIETER

Introduction

The proper replication and segregation of chromosomes in a mitotic division requires the correct execution of a large number of biochemical reactions. The substrate in these reactions, the chromosome, is complex, exhibiting morphological and functional differentiation along its length as evidenced by the presence of specialized domains such as centromeres and telomeres. The cellular machinery that catalyzes these reactions is also complex, consisting of many trans-acting factors often assembled in complicated structures, for example, the spindle. The intricate nature of the substrate and machinery apparently assure that the reactions are executed with extremely high fidelity. For example, errors in chromosome transmission in the yeast, *Saccharomyces cerevisiae*, occur as infrequently as once per 10^5 cell divisions.[1,2]

An understanding of mitotic chromosome transmission at the molecular level will only be achieved when one knows how each chromosomal domain and component of the cellular machinery functions in the individual steps of the process. This functional dissection of chromosome transmission will require genetic as well as biochemical approaches, in particular the isolation and characterization of mutants that are defective in the process. The isolation of these mutants is not trivial because replication and segregation are essential processes and mutations which destroy them will be lethal to the cell. However, the genetic analysis of chromosome transmission in the yeast, *S. cerevisiae*, is feasible because the fidelity of chromosome transmission exhibited by wild-type strains is much greater than the fidelity needed for viability. Thus mutations that dramatically reduce but do not destroy the fidelity of the process are viable in yeast. These mutations will include hypomorphs, leaky mutations in functions essential for chromosome transmission, or null mutations in functions that contribute to fidelity but are not essential for chromosome transmission.

Given that yeast mutants with altered chromosome transmission are

[1] L. H. Hartwell, S. K. Dutcher, J. S. Wood, and B. Garvik, *Recent Adv. Yeast Mol. Biol.* **1**, 28 (1982).
[2] M. S. Esposito, D. T. Maleas, K. A. Bjornstad, and C. V. Brushi, *Curr. Top. Genet.* **6**, 5 (1982).

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